METHOD

Functional siRNA expression from transfected PCR products

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ABSTRACT

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) induces the postranscriptional degradation of homologous transcripts. RNAi can be initiated by exposing cells to dsRNA either via transfection or endogenous expression. In mammalian systems, the sequence-specific RNAi effect has been observed by expression of 21-23 base transcripts capable of forming duplexes, or via expression of short hairpin RNAs. We describe here a facile PCR based strategy for rapid synthesis of siRNA expression units and their testing in mammalian cells. The siRNA expression constructs are constructed by PCR, and the PCR products are directly transfected into mammalian cells resulting in functional expression of siRNAs. This approach should prove useful for identification of optimal siRNA-target combinations and for multiplexing siRNA expression in mammalian cells.

Keywords: message down-regulation; Pol III expression; short hairpin RNAs; small interfering RNAs

INTRODUCTION

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) induces the postranscriptional degradation of homologous transcripts, and has been observed in variety of organisms including plants, fungi, insects, protozans, and mammals (Bernstein et al., 2001b; Elbashir et al., 2001a, 2001b; Moss, 2001). RNAi is initiated by exposing cells to dsRNA either via transfection or endogenous expression. Double-stranded RNAs are processed into 21- to 23-nt double-stranded fragments known as small interfering RNAs (siRNA; Elbashir et al., 2001a, 2001b). These siRNAs form a complex known as the RNA-induced silencing complex or RISC (Bernstein et al., 2001a; Hammond et al., 2001), which functions in homologous target RNA destruction. In mammalian systems, the sequence-specific RNAi effect has been observed by introduction of siRNAs either via transfection or endogenous expression of 21–23-base transcripts or longer hairpin RNAs. Use of siRNAs evades the dsRNA-induced interferon and PKR pathways that lead to nonspecific inhibition of gene expression (Elbashir et al., 2001a).

Recently, several groups have demonstrated that siR-NAs can be effectively transcribed by Pol III promoters in human cells and elicit target-specific mRNA degradation (Ketting et al., 2001; Brummelkamp et al., 2002; Lee et al., 2002; Miyagishi & Taira, 2002; Paul et al., 2002). These siRNA-encoded genes have been transiently transfected into human cells using plasmid or episomal viral backbones for delivery. Transient siRNA expression can be useful for rapid phenotypic determinations preliminary to making constructs designed to obtain long-term siRNA expression. Of particular interest is the fact that not all sites along a given mRNA are equally sensitive to siRNA-mediated down-regulation (Elbashir et al., 2001a; Holen et al., 2002; Lee et al., 2002; Yu et al., 2002). There are at this time no rules governing siRNA target site selection for a given mRNA target. We have previously taken advantage of an oligo-nucleotide/RNAse H procedure in cell extracts on native mRNA transcripts originally designed for identifying ribozyme accessible sites, and have found that this approach can be applied to siRNA site accessibility as well (Lee et al., 2002). Unfortunately, this process can be time consuming, and, in the end, it is still necessary to synthesize the siRNA genes for final testing. Cloning the siRNAs in the expression vector of choice can also be a time-consuming process, although, if the unusually compact H1 promoter is used, it can be facilitated

by the annealing and extension of two oligonucleotides containing the hairpin and promoter sequences (McManus et al., 2002). In this report, we describe a PCR-based approach for rapid synthesis of Pol III promoter–siRNA gene constructs and their subsequent transfection into cells. This procedure can be utilized for the facile screening of siRNA encoding genes to identify those with the best functional activity for a given target. The PCR products are used directly, without subsequent cloning, by transfecting them into cells followed by functional assays. The method described here can also be used for screening siRNA gene libraries. The method is fast and inexpensive, allowing several different siRNA gene candidates to be rapidly screened for efficacy. Our approach can be utilized with siRNAs expressed independently from Pol III promoters or siRNAs that are transcribed as hairpin precursors.

RESULTS AND DISCUSSION

The procedure for the PCR approach is depicted schematically in Figure 1. A universal primer that is comple- mentary to the 5' end of the human U6 promoter is used in a PCR reaction along with a primer(s) comple- mentary to the 3' end of the promoter that harbors appended sequences that are complementary to the sense or antisense siRNA genes (Fig. 1A). The sense or the antisense sequences are followed by a stretch of six deoxyadenosines (Ter) and by a short additional "stuffer-tag" sequence that includes a restriction site for possible cloning at a later stage. The resulting PCR products include the U6 promoter sequence, the sense or antisense sequence, a terminator sequence, and the stuffer-tag sequence at the 3' terminus of the product. Alternatively, both the sense and antisense sequences can be included in the same cassette (Fig. 1B). In this case a 9-nt loop is inserted between the sense and antisense siRNA sequences. The resulting single PCR product includes the U6 promoter, the sense and anti- sense siRNAs in the form of a stem-loop, the termina- tor sequence, and the tag sequence. To construct this cassette two 3' primers are used. The first PCR reac- tion employs the 5' U6 universal primer and a 3' primer complementary to 20 nt of the U6 promoter, followed
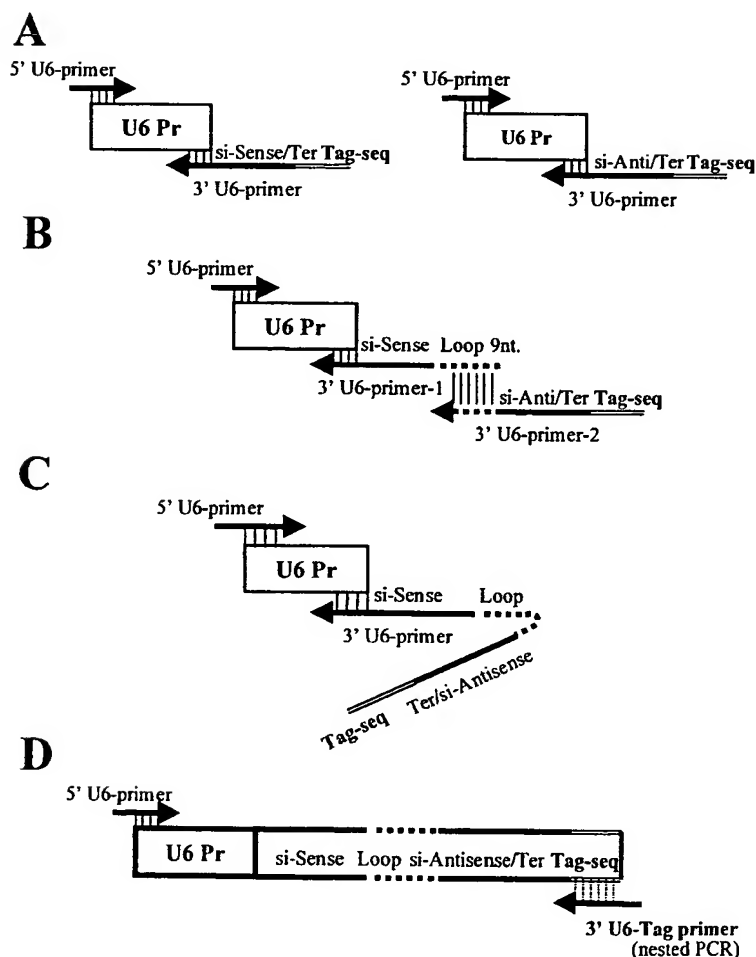
FIGURE 1. Schematic representation of the PCR strategy used to yield U6 transcription cassettes ex- pressing siRNAs. The 5' PCR primer is complemen- tary to the 5' end of the U6 promoter and is standard for all PCR reactions. A: The 3' PCR primer is com- plementary to sequences at the 3' end of the U6 promoter followed by either the sense or antisense sequences, a stretch of four to six deoxyadenosines (Ter), and an additional stuffer-tag sequence. The adenosines are the termination signal for the U6 Pol III promoter; therefore, any sequence added after this signal will not be transcribed by the Pol III polymer- ase and will not be part of the siRNA. B: The sense and antisense sequences are linked by a 9-nt loop and are inserted in the cassette by a two-step PCR reaction (see text). C: The sense and antisense se- quences linked by a 9-nt loop and followed by the stretch of adenosines and by the Tag sequences are included in a single 3' primer. D: Complete PCR ex- pression cassette obtained by the PCR reaction. To amplify and identify functional siRNAs from the trans- fected cells, or to increase the yield of the PCR prod- uct shown in B, a nested PCR can be performed using the universal 5' U6 primer and a 3' primer com- plementary to the Tag sequence (also standard), as indicated in the figure.

by sequences complementary to the sense and the 9-nt loop (Fig. 1B). One microliter of this first reaction is reamplified in a second PCR reaction that employs the same 5' U6 primer and a 3' primer harboring sequences complementary to the 9-nt loop appended to the antisense strand, Ter, and stuffer-tag sequence (Fig. 1B). The resulting PCR products include the U6 promoter and the sense and antisense coding sequences, followed by the Pol III terminator sequence and the stuffer-tag sequence (Fig. 1D). It is also possible to use a one-step PCR reaction with a single 3' primer that harbors the sense, loop antisense, Ter, and stuffer-tag sequences (Fig. 1C). Although this considerably long and structured 3' PCR primer could in some cases cause difficulties, this approach may be preferable for direct transfection of PCR products since it eliminates the likelihood of PCR mutations in the siRNA sequence.

In our experience, the PCR conditions are relatively standard for all siRNA genes because the regions complementary to the U6 promoter do not change. For the construction of several cassettes, we found that optimal amplification was achieved in each case using 1 min for each PCR step and 55 °C as the annealing temperature. For direct transfections and testing of the PCR-amplified siRNA genes, the 5' termini of the PCR primers were phosphorylated using DNA polynucleotide kinase and nonradioactive ATP. We have observed that this modification results in enhanced expression of the PCR products, perhaps by stabilizing them intracellularly.

Once the PCR reaction is completed, the products are column purified from the primers. The purified products can be applied to cells following cationic liposome encapsidation and/or standard transfection procedures. Intracellular expression of the transfected PCR products was easily detected by northern blotting analyses (Fig. 3A), demonstrating good transfection efficiency.

To test the PCR-amplified siRNA encoding DNA genes for their inhibitory efficacy in cells, we used an HIV-1 *rev* sequence fused to the enhanced green fluorescent protein (EGFP) coding sequence (Lee et al., 2002). This construct was inserted in the Ecdysone-inducible pIND vector system (Invitrogen). We also utilize a 293 cell line that stably expresses the *trans*-activator for the inducible promoter (Lee et al., 2002). Use of this system results in strong EGFP expression following addition of Ponasterone A (Invitrogen) to the culture media (Fig. 3A).

A stable cell line expressing both the *trans*-activator and target constructs may be preferable when multiple siRNA genes are being tested, but cotransfection with a target-EGFP fusion construct provides a rapid and sensitive test for siRNA efficacy. Target sequence cDNAs can be readily cloned into this inducible vector system to create the desired EGFP fusion. Utilizing this system, an effective siRNA expressed from the PCR product will inhibit EGFP expression allowing either FACS or microscopic-based analyses of siRNA function.

To test the PCR approach, U6 cassettes encoding either the sense or antisense siRNA genes (Fig. 1A) or a hairpin construct encoding both the sense and antisense siRNAs (Fig. 1C) were amplified. The PCR products were column purified. The PCR products were cotransfected with the inducible *rev*-EGFP fusion construct into the Ecdysone transactivator-expressing cell line. At 48 h posttransfection, Ponasterone A was added to the culture to induce target mRNA expression. Using this system, we were able to detect a strong and specific down-regulation of EGFP expression by the siRNAs 12 h postinduction (Fig. 2). Transfection of a U6 expression cassette expressing only the sense (Fig. 2B), the antisense (Fig. 2C), or an irrelevant siRNAs (not shown) had no effect on expression of EFGP. However, when cassettes expressing the sense and antisense siRNAs were cotransfected with the target, or when a single cassette containing the hairpin siRNA gene was used, a specific and effective down-regulation of the target was detected (Fig. 2D, E). The best and most reproducible inhibition (nearly 90%) was obtained with the hairpin siRNA-expressing cassette. These results were reproduced independently five times. The selected length and sequence of the 9-base loop (UUUGUG UAG) used for these experiments is based on comparisons of loops found in several micro-RNA precursors. We find that this 9-base loop is more effective than other lengths and sequences (data not shown). When using this loop, it is important that the siRNA sense strand does not contain a U as the 3' base because this would create a stretch of 4 uridines that could serve as a Pol III terminator element.

The results presented here indicate that this transfection-PCR methodology can be used to rapidly test siRNA targeting and function in cells. One critical element in the design of effective siRNAs is the selection of siRNA/target sequence combinations that yield the best inhibitory activity. This can be accomplished using siRNAs and transfection procedures, but this can be a costly and time-consuming step. By utilizing the PCR strategy, several siRNA genes can be simultaneously tested in a single transfection experiment. Noteworthy is the on-line tool, made available by Hannon's laboratory, which allows the user to input a gene sequence and receive the hairpin-specific PCR primers as output (http://www.cshl.org/public/SCIENCE/ hannon.html). To facilitate the identification of functional siRNA genes, we have inserted a stuffer-tag sequence directly after the Pol III transcription terminator (see Fig. 1). By utilizing this tag, a transfected PCR cassette can be amplified from transfected cells and the siRNA sequence subsequently identified (Fig. 1C). The tag sequence starts with the 6 Ts of the Terminator sequence followed by a restriction site that can be used for subsequent cloning, and a stuffer of six extra nucleotides (for a total of 18 nt).
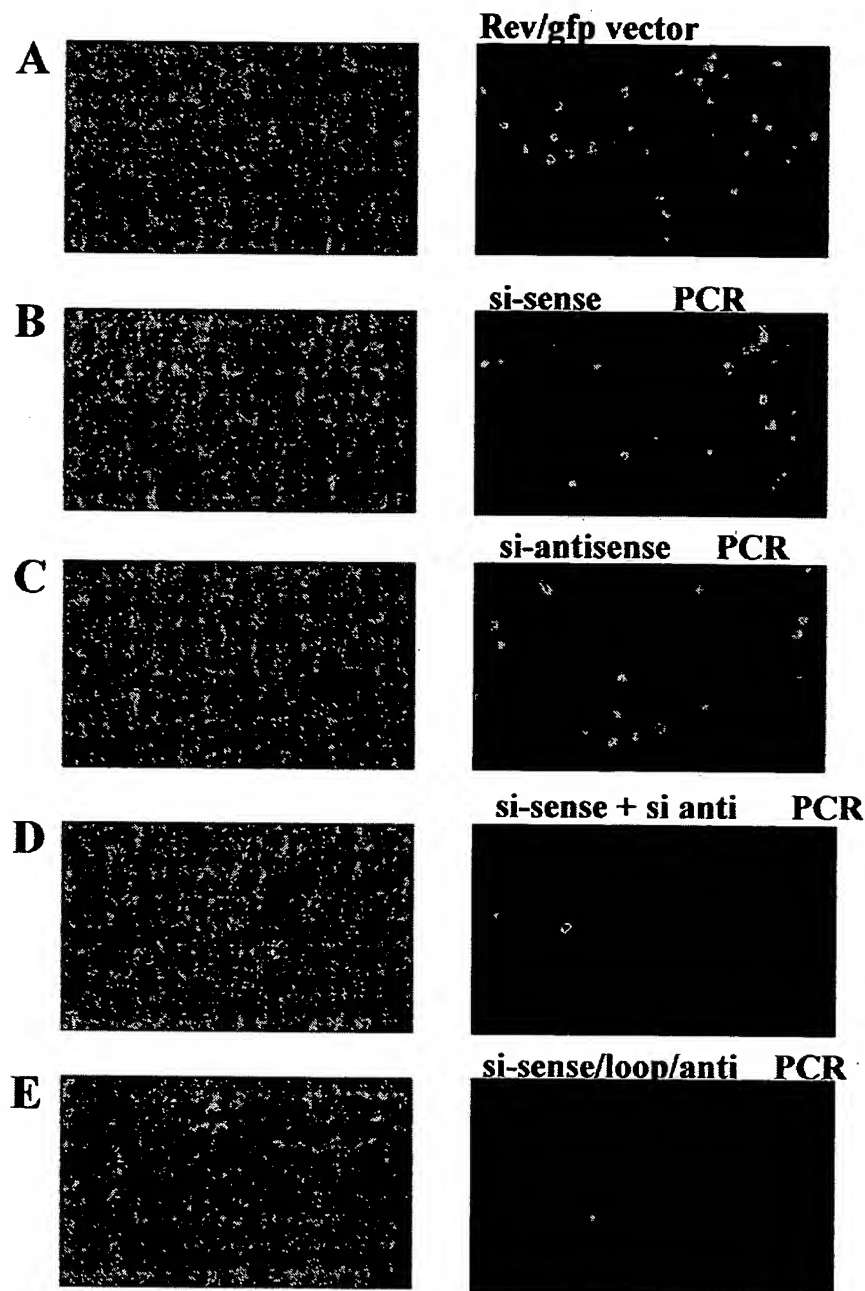
FIGURE 2. Inhibition of EGFP expression using siRNA-containing PCR cassettes transfected in 293 cells. The PCR cassettes containing the sense, antisense, or both sense and antisense siRNAs were cotransfected with the target construct into 293 cells expressing the Ecdysone *trans*-activator. The HIV *rev* target is fused to the green fluorescent protein mRNA, which is expressed from an inducible promoter. After adding Ponasterone A, EGFP expression can be detected in the control cells (**A**), but not in cells transfected with either a mixture of sense and antisense siRNA expressing PCR products (**D**), or with the PCR cassette expressing the hairpin construct (**E**). **B** and **C** depict cotransfection of cells with target and PCR cassettes expressing sense alone (**B**) or antisense alone (**C**). The Rev-EGFP protein is primarily in the cell nucleolus as a consequence of the nucleolar localizing signal in the Rev portion. The right panels show the analyzed cells under a bright field.

To test the feasibility of this strategy, 15 ng of the PCR-amplified gene encoding the siRNA hairpin targeting the *rev* site, along with 15 ng of an irrelevant siRNA PCR product were cotransfected with the inducible target-EGFP cassette into the 293 cell line expressing the *trans*-activator. Forty-eight hours post-transfection, Ponasterone A was added to induce EGFP expression, and after 12 h the EGFP negative and pos-

itive cells were FACS sorted. The cell pellets from both the EGFP positive and negative sorts were collected by centrifugation, lysed overnight in lysis buffer, and the DNAs amplified directly by PCR utilizing the appropriate primer sets. For this experiment, we used two different 3′ primers that discriminate between the two different siRNA-encoding DNA cassettes. Our expectations were that the nonfunctional siRNA expression

cassette should be detectable by PCR amplification in both cell fractions, whereas the functional siRNA expression cassette would only be detectable in the EFGP negative fraction, as its products would have functionally down-regulated EGFP expression. The results from two independent experiments are shown in Figure 3B, C. In both cases, the nonfunctional siRNA-encoding gene was PCR amplified from all fractions (Fig. 3B),
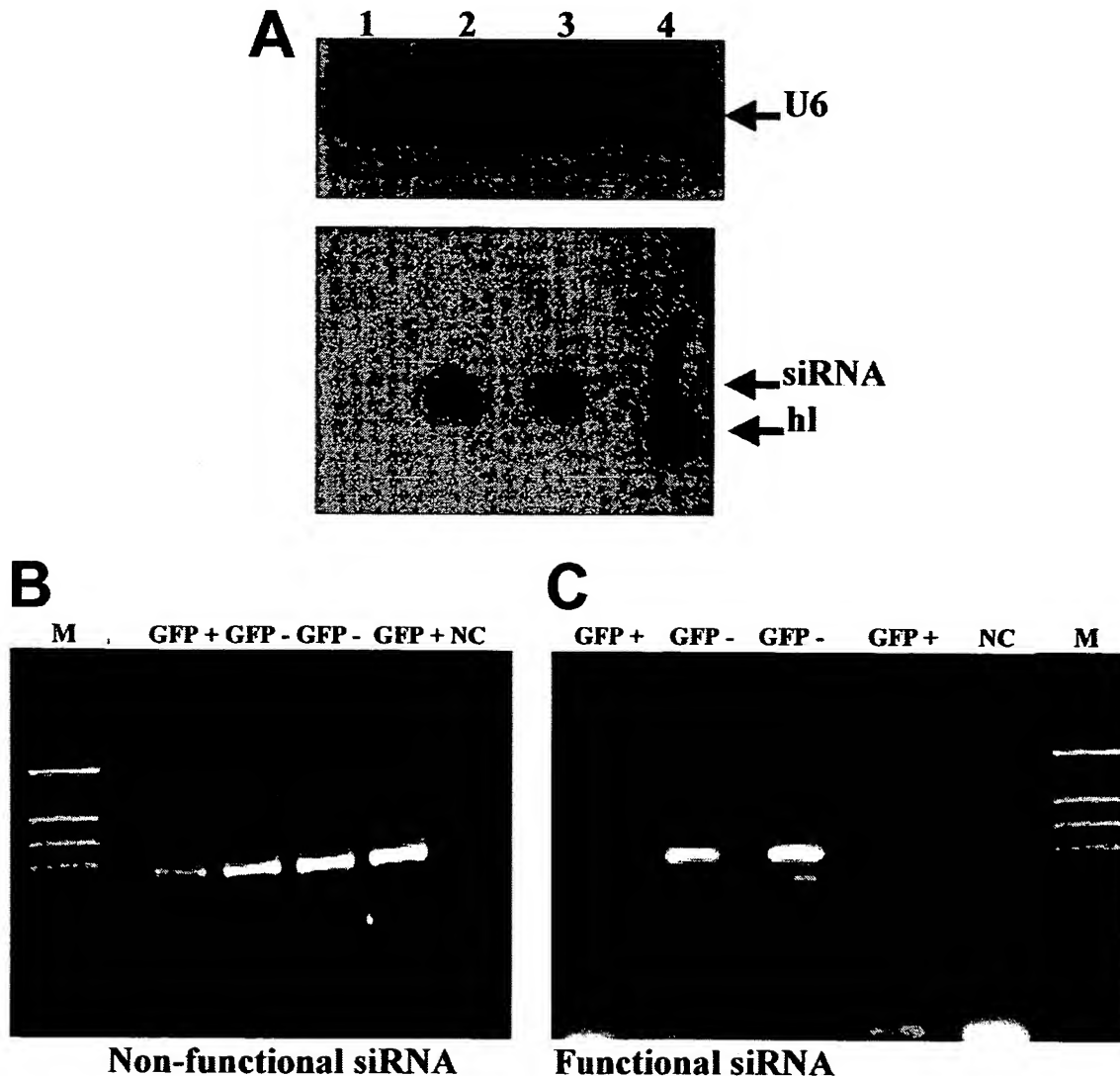
FIGURE 3. Detection of siRNAs and PCR amplification of siRNA encoding DNAs from transfected cells. A: Northern gel analyses of siRNAs expressed from PCR products transfected in A293 cells. Lane 1: cells transfected with the EFGP target construct alone; lane 2: cells transfected with antisense encoding construct alone; lane 3: cells cotransfected with antisense and sense encoding constructs; lane 4: cells transfected with hairpin expression construct. The probe is complementary to the antisense. In lanes 2–4, the siRNA-encoding DNA constructs were cotransfected with the inducible EGFP construct. Note that the hairpin product (hl) is slightly smaller than the individually expressed siRNAs, demonstrating processing of the hairpin loop B and C. PCR amplification of transfected PCR constructs. B: PCR amplification of nonspecific siRNA encoding DNA from FACS sorted EFGP positive and negative cells. The nonfunctional construct is detected in all cell fractions. Lanes 1 and 4 show the amplification results from the EGFP positive fractions. Lanes 2 and 3 show the amplification results from the EGFP negative fractions. C: PCR amplification of functional hairpin expression construct from FACS-sorted, EGFP-expressing, and nonexpressing cells. The amplification results show the presence of the functional siRNA only in the EGFP negative fractions (lanes 2 and 3). In lane 4 there is a small amount of amplified product, perhaps derived from some contaminating EGFP negative cells. The M indicates the *Hpa*II digested Bluescript SK marker.

whereas the functional siRNA-encoding expression construct was primarily detected in the EGFP negative cell fractions (Fig. 3C).

Using variations of the above approach, it should be possible to create several expression cassettes and simultaneously screen for siRNA-sensitive target sites on any given mRNA. If it is feasible to fuse the target sequence to EFGP or a similar reporter, screening can be rapidly accomplished via FACS analyses and sorting. This strategy can also utilized for endogenous targets when there is a positive selection or a FACS sortable phenotype available. In conclusion, this PCR strategy allows a rapid and inexpensive approach for intracellular expression of siRNAs and subsequent testing of target site sensitivity to down-regulation by siRNAs.

MATERIALS AND METHODS

Target construction and location of the siRNA target site

The target used in our experiment is the HIV-*rev* sequence followed by the EGFP gene cloned in the pIND-inducible vector (Invitrogen) as previously described (Lee et al., 2002). The selection of the accessible target site for the siRNA was based on previous work from our laboratory (A. Poggi & J.J. Rossi, unpubl. results) and was shown to be an effective siRNA target using the U6 expression system (Lee et al., 2002). The sequence of the target site is 5'-GCCTGTGCCT CTTCAGCTACC-3', which is located 213 nt downstream of the *rev*-AUG start codon.

Polymerase chain reaction

PCR reactions were performed using a plasmid containing the human U6 promoter as template. The 5' oligonucleotide (5'U6 universal primer) is complementary to 29 nt at the 5' end of the U6 promoter (bold italics) 5'-ATCGCAGATCT**GG ATCCAAGGTCGGGCAGGAAGAGGGCCT**-3' and was used for all PCR steps. The 3' oligonucleotides, which contain either the sense, antisense, or both siRNAs, are depicted in Figure 1 and are described in the text. The last 20 nt at the 3' end of all 3' PCR primers are complementary to the last 20 nt of the U6 promoter, which is: 5'-**GTGGAAAGGACGAAACA CCG**-3'. All PCR reactions were carried out as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 30 cycles. The use of Vent Polymerase is recommended. Prior to amplification, the PCR primers were kinased for 30 min with nonradioactive ATP using T4DNA ligase buffer and 1 μL of kinase enzyme (New England Biolabs). The kinased oligos were purified on G50 columns (Amersham Pharmacia Biotech) as suggested by the manufacturer, prior to using them in the PCR reactions. The PCR products were also purified using the QIAquick PCR purification Kit (Qiagen).

The sequences of the siRNA encoding oligos are:

1. Sense for siRNA rev: 5'-CGAAAAGGCCTAAAAAA*GGTA GCTGAAGAGGCACAGGC*GGTGTTTCGTCCTTTCCAC AAGATATATAA-3';

2. Antisense for siRNA rev: 5'-CGAAAAGGCCTAAAAAA*GC CTGTGCCTCTTCAGCTACC*GGTGTTTCGTCCTTTCCA CAAGATATATAA-3';

3. Hairpin siRNA oligo 1-sense: 5'-TACACAAA*GGTAGCTG AAGAGGCACAGGC*GGTGTTTCGTCCTTTCCACAAGA TATATAA-3';

4. Hairpin siRNA oligo 2-antisense: 5'-CGAAAAGGCCTAA AAAA*GCCTGTGCCTCTTCAGCTACCC*TACACAAAGG-3'.

The italicized sequences are the siRNA encoding sequences.

Cell lines and culture conditions

293 cells were grown in DMEM (Irvine Scientific, Santa Ana, California) supplemented with 10% fetal calf serum (Irvine Scientific), 1 mM L-glutamine, and 100 U/mL of penicillin/ streptomycin. The Ecdysone-inducible stable A293 clone has been previously described (Lee et al., 2002), and was maintained in DMEM containing 100 μg/mL of Zeocin (Invitrogen).

Transfection conditions for siRNA-PCR products

Two hundred fifty nanograms of the target plasmid were cotransfected with (1) 50 ng of the PCR cassette expressing the sense and/or 50 ng of the cassette expressing the antisense siRNA, or (2) 100 ng of the single cassette expressing both the sense and antisense linked by a 9-nt loop. As few as 25 ng of the stem loop siRNA was effective in blocking target expression. A U6 promoter PCR cassette containing an irrelevant siRNA designed to target a site in the *gag*-HIV-1 gene was used as an additional control and did not result in any effect on target expression (not shown).

To facilitate the transfection of the small amounts of PCR amplified DNA, 400 ng of Bluescript plasmid were added to each reaction to serve as carrier. Five micromolar Ponasterone A was added to the culture media 48 h after transfection, and the cells were analyzed for EGFP expression 12 h following induction. Transfections were performed in 6-well plates using Lipofectamine Plus™ reagent (Life Technologies, Gibco BRL) as described by the manufacturer. For microscope imaging, cells were grown and transfected on glass coverslips treated with 0.5% gelatin (Sigma). At 12 h postinduction, the coverslips were lifted from the 6-well plate and treated for 10 min at room temperature with 4% PFA for cell fixation. Down-regulation of the *rev*-EGFP mRNA was quantitated by FACS analyses.

Northern analyses

Total RNA was isolated using RNA STAT-60 (TEL-TEST·B Inc., Friendswood, Texas) according to the manufacturer's instructions. Five micrograms of total RNA were fractionated in 8 M-6% PAGE, and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech). A ^{32}P-radiolabeled 21-mer probe complementary to the siRNA-antisense was used for the hybridization reactions, which were performed for 16 h at 37°C. A 21-mer DNA oligonucleotide was electro-

phoresed alongside the RNA samples and used as a size marker and hybridization control (not shown).

Direct amplification of siRNAs
from cell lysates

EGFP-negative and -positive cell fractions were collected by FACS sorting. The cell pellets were recovered immediately by centrifugation of the sorted fractions. The pellets were lysed in 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.25 mM $MgCl_2$, 0.45% NP40, 0.45% Tween, and 0.75 μg/μL Proteinase K at 37 °C overnight. After 10 min heat inactivation at 95 °C, 3 μL of the cell lysates were used directly in the PCR reactions. The oligonucleotides used in the PCR reactions to distinguish the functional and nonfunctional siRNA cassettes were the 5' U6 universal primer for both cassettes with either the hairpin siRNA oligo 2-antisense for the functional cassette or the 3' si-*gag*-antisense primer (5'-GATAAGATCTCCTAGGA AAAAAGGAGAGAGATGGGTGCGAGAG-3') for the irrelevant cassette.

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